

A Visual Reporter System for Virus-Induced Gene Silencing in Tomato Fruit Based on Anthocyanin Accumulation^{1[C][W]}

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Virus-induced gene silencing (VIGS) is a powerful tool for reverse genetics in tomato (*Solanum lycopersicum*). However, the irregular distribution of the effects of VIGS hampers the identification and quantification of nonvisual phenotypes. To overcome this limitation, a visually traceable VIGS system was developed for fruit, comprising two elements: (1) a transgenic tomato line (*Del/Ros1*) expressing *Antirrhinum majus* Delila and Rosea1 transcription factors under the control of the fruit-specific E8 promoter, showing a purple-fruited, anthocyanin-rich phenotype; and (2) a modified tobacco rattle virus VIGS vector incorporating partial *Rosea1* and *Delila* sequences, which was shown to restore the red-fruited phenotype upon agroinjection in *Del/Ros1* plants. Dissection of silenced areas for subsequent chemometric analysis successfully identified the relevant metabolites underlying gene function for three tomato genes, *phytoene desaturase*, *TomloxC*, and *SLOD1*, used for proof of concept. The C-6 aldehydes derived from lipid 13-hydroperoxidation were found to be the volatile compounds most severely affected by *TomloxC* silencing, whereas geranial and 6-methyl-5-hepten-2-one were identified as the volatiles most severely reduced by *phytoene desaturase* silencing in ripening fruit. In a third example, silencing of *SLOD1*, a tomato homolog of the *ODORANT1* gene encoding a myb transcription factor, which regulates benzenoid metabolism in petunia (*Petunia hybrida*) flowers, resulted in a sharp accumulation of benzaldehyde in tomato fruit. Together, these results indicate that fruit VIGS, enhanced by anthocyanin monitoring, can be a powerful tool for reverse genetics in the study of the metabolic networks operating during fruit ripening.

Virus-induced gene silencing (VIGS) is a powerful tool for functional gene analysis in plants (Dinesh-Kumar et al., 2007). VIGS relies on transient as opposed to stable plant transformation; the spread of the silencing phenotype is governed by the speed of transmission of the silencing signal to new organs or within organs and, consequently, silenced tissues can be obtained rapidly. VIGS has been shown to work in a broad spectrum of recipient plant species, and technical procedures are relatively simple and easily adapted

to high-throughput strategies; consequently, VIGS has become a popular reverse genetics tool in both model species and crops (Burch-Smith et al., 2006; Wang et al., 2006, 2007; Nelson et al., 2007; Wege et al., 2007; Zhang et al., 2009).

All of these factors make VIGS particularly suitable for the study of gene function in tomato (*Solanum lycopersicum*) fruit, where the enormous amount of data arising from genomic projects requires rapid, high-throughput methods to link gene functions to phenotypes. Particularly important for tomato is to understand the genetic basis of the metabolic pathways that operate in fruit, especially those active during the ripening process, which influence the nutritional value and organoleptic features of the crop. It has been shown recently that successful “local” silencing effects can be obtained by direct inoculation (agroinjection) of VIGS (or RNA interference) constructs into developing fruit (Fu et al., 2005; Hoffmann et al., 2006; Orzaez et al., 2006). This methodology speeds up the experimental procedure appreciably. Indeed, confined VIGS signals permit shifting from the classical “one plant-one biological replicate” approach to “one organ-one biological replicate,” increasing the throughput of VIGS.

However, the use of VIGS for functional analysis of genes involved in fruit metabolism carries appreciable technical limitations. With the exception of hypersus-

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ceptible *Nicotiana benthamiana*, the penetration of VIGS phenotypes in most plant species is only partial and shows a patchy tissue distribution. *Tobacco rattle virus* (TRV), for example, which drives a highly efficient VIGS in all newly developed organs of *N. benthamiana* (Ruiz et al., 1998; Ratcliff et al., 2001; Dong et al., 2007), works less efficiently in potato (*Solanum tuberosum*) and tomato (Liu et al., 2002; Brigneti et al., 2004; Ryu et al., 2004), probably because parts of the plant remain poorly silenced or not silenced at all (Rotenberg et al., 2006; Senthil-Kumar et al., 2007). Tomato plants inoculated at the cotyledon stages show silenced symptoms in about half of their fruit. In most cases, these fruit show partial silencing, with silenced sectors representing only 10% to 30% of the fruit surface. The percentage of silenced fruit reaches 90% when tomatoes are agroinjected directly in the fruit, although the extent of silencing is still partial and highly variable from fruit to fruit (average silenced surface = 35%; Orzaez et al., 2006). Partial VIGS penetration does not impede the screening of visually identifiable phenotypes but becomes a serious limitation for the study of nonvisual, quantitative traits (e.g. effects on metabolite levels).

A strategy to compensate for the lack of uniformity of VIGS would incorporate an internal reference that monitors the levels of silencing. The presence of such a reporter would allow the dissection of silenced from nonsilenced tissues, thus increasing the sensitivity of downstream analysis. Ideally, an internal reporter for VIGS should be silenced in the same cell/regions as the gene of interest (GOI), easily scorable (visually), and its depletion should have a limited impact on the process of interest.

To establish a high-throughput platform for quantitative evaluation of gene function using VIGS in tomato fruit, we considered several potential VIGS markers. Endogenous genes involved in pigment accumulation, such as those encoding phytoene desaturase (PDS), have limited use. *pds* silencing, which renders yellow, lycopene-depleted fruit (Liu et al., 2002; Orzaez et al., 2006), blocks carotenoid biosynthesis and consequently affects the production of compounds involved in color, nutritional, and flavor value in tomato fruit. Transgenes encoding fluorescent proteins such as GFP have been widely used in model species for monitoring gene silencing, but GFP-producing tomato fruit offer few advantages for VIGS, since they require UV light for manipulation and fluorescence is often too weak to identify clear boundaries between silenced and nonsilenced tissues.

Recently, the use of internal markers for VIGS based on pigment accumulation in petunia (*Petunia hybrida*), soybean (*Glycine max*), and tomato has proven very successful for empowering the method as a reverse genetics tool (Chen et al., 2004b; Nagamatsu et al., 2007; Spitzer et al., 2007; Jiang et al., 2008). Tomato introgression lines accumulating anthocyanins in their fruit have been described, but this accumulation is restricted to the epidermis; therefore, the use of these

lines is restricted to analysis of gene function in this tissue. An alternative is to use a transgenic approach that has the advantage of being readily applicable to different genetic backgrounds. The anthocyanin branch of flavonoid biosynthesis is activated in *Antirrhinum majus* flowers by two transcription factors, *Rosea1* and *Delila* (Schwinn et al., 2006). Tomato plants ectopically expressing *Rosea1* and *Delila* in their fruit under the control of the ripening-induced E8 promoter have been shown to accumulate high levels of anthocyanin pigments, not only in the peel but also in the flesh (pericarp; Butelli et al., 2008). Anthocyanin accumulation in *Del/Ros1* transgenic fruit results from the activation of almost all of the genes encoding anthocyanin biosynthetic enzymes, including genes required for side chain modification and two genes involved in vacuolar import.

The easily scorable transgenic phenotype of *Del/Ros1* fruit prompted us to investigate its use as a visual reporter system for VIGS in tomato. We found that silencing of the transgene module restored the original wild-type phenotype. Second, the requirements for the simultaneous silencing of the *Del/Ros1* marker module and a target GOI were investigated; simultaneous silencing takes place consistently only when both sequences are integrated together in the same viral genome. Third, the sensitivity of the *Del/Ros1* VIGS marker system for functional chemometric studies was tested with two model genes whose effects on volatile emissions from tomato fruit have already been described: *TomloxC*, involved in the biosynthesis of fatty acid-derived aromas (Chen et al., 2004a), and *pds*, involved in the production of lycopene-derived volatiles. In both examples, analysis of gas chromatography-mass spectrometry (GC-MS) data sets provided by anthocyanin-monitored fruit VIGS (AMFV) correctly identified the expected changes in volatile profiles. Finally, the function of the novel tomato gene *SIODO1* was also tested using AMFV. *SIODO1* is a tomato homolog of the petunia myb factor ODORANT1, which positively regulates benzenoid volatile levels in flowers. Silencing of *SIODO1* using AMFV detected, as expected, severe changes in the profile of phenolic compounds in the fruit. *SIODO1*-silenced fruit pericarp showed reduced levels of methyl salicylate and guaiacol. Interestingly, *SIODO1*-silenced tissues also showed very high levels of the precursor benzaldehyde, suggesting that *SIODO1* is not only a general activator of the pathway in tomato but also plays a role in modulating the relative abundance of different benzenoid compounds in the fruit.

RESULTS

Virus-Induced Silencing of *Del/Ros1* Prevents the Purple-Fruited Phenotype of *Del/Ros1* Plants

The accumulation of purple anthocyanin compounds in the fruit of *Del/Ros1* MicroTom (MT) plants

(Butelli et al., 2008) used in this paper is directed by the fruit-specific, ethylene-regulated E8 promoter. Consequently, tomatoes at the mature green stage start to accumulate purple compounds approximately 35 d after pollination, depending on the light and temperature conditions. Initially, purple pigments accumulate on a green background, due to the presence of chlorophyll, giving a brown-purple coloration. Later in the process, carotenoid-derived pigments, initially yellow and later the red lycopene, accumulate and replace the chlorophyll. Carotenoid-dominated pigmentation is rapidly masked by anthocyanin accumulation, and fruit rapidly turn dark purple as ripening proceeds (Supplemental Fig. S1A).

To evaluate the possible use of purple-fruited phenotype as a visual marker, the ability of VIGS to prevent anthocyanin accumulation in *Del/Ros1* fruit was tested. A DNA fragment containing *Delila* (130 bp) and *Rosea1* (130 bp) gene fragments fused by overlapping PCR was incorporated into pTRV2 vector (pTRV2_DR). The construct was either agroinfiltrated in plant cotyledons or agroinjected in fruit at 25 to 30 d after anthesis. In both cases, red sectors in the background of purple were clearly visible upon fruit ripening (Supplemental Fig. S1B). These results confirmed that accumulation of anthocyanins promoted by the *Del/Ros1* module could be prevented by VIGS, therefore producing a monitorable phenotype.

Tandem Constructs of *Del/Ros1* and a GOI Are Needed for Efficient Cosilencing

To be useful as a monitoring system, the silencing of the *Del/Ros1* module and, consequently, the prevention of purple pigmentation needs to be spatially localized in the same region in which maximum silencing of the GOI is taking place. We used *pds* as a model GOI to test the requirements for simultaneous cosilencing in *Del/Ros1* tomato fruit. The silencing of *pds* in a wild-type background was easy to score because the lack of accumulation of the red pigment lycopene results in yellowish fruit. First, the possibility of achieving cosilencing by codelivery of independent pTRV2 constructs was assayed. In the event of cosilencing, yellow sectors should be observable in the silenced fruit as a result of blocking both lycopene and anthocyanin accumulation. As shown in Figure 1A, the fruit had small red sectors corresponding to areas with strongly silenced anthocyanin accumulation and unaffected lycopene accumulation. Red sectors were surrounded by large areas of unmodified purple tissue. The strong accumulation of anthocyanins in the ripening fruit, which predominates over the development of red color, prevented us from identifying those sectors in the fruit silenced only for *pds* (depleted of lycopene content) visually. However, the early accumulation of anthocyanins in the green fruit provided a window of opportunity for observing *pds* silencing alone. In the nonsilenced fruit, purple anthocyanins accumulate in the green tissues, rendering mixed green-purple colors

(Supplemental Fig. S1A). As shown in Figure 1B, *pds* silencing on its own was evidenced by the presence of unripe fruit with a light purple coloration, which resulted from anthocyanin accumulation (*Del/Ros1* nonsilenced) over a white, chlorophyll-depleted (*pds*-silenced) background in systemically silenced plants. Therefore, simultaneous agrodelivery was not sufficient to ensure cosilencing in the tomato *Del/Ros1* system.

We then assayed the efficiency of cosilencing when *Del/Ros1* and GOI fragments were cloned in tandem within the same viral construct and consequently delivered together. To facilitate high-throughput cloning of subsequent GOIs, the pTRV2_DR vector was engineered to contain a Gateway recombination cassette (pTRV2_DR_GW) and, as a proof of concept, the 409-bp *pds* fragment was inserted by homologous recombination, producing the pTRV2_DR_PDS construct. This construct was agroinfiltrated into *Del/Ros1* tomato plants at the cotyledon stage (systemic VIGS), and the effect on fruit color was monitored. Some of the resulting phenotypes are shown in Figure 1C. As can be observed, only yellow (indicative of effective cosilencing) but no red (indicative of unilateral silencing of *Del/Ros1*) sectors were observed in the fruit. Moreover, during development, all fruit showed either green or green-purple combinations but no light purple (purple on white) sectors, confirming efficient cosilencing (Fig. 1D).

The correlation between the extent of silencing (measured as mRNA levels) and the anthocyanin content in the silenced fruit was investigated. *Del/Ros1* fruit were agroinjected 4 weeks after anthesis with pTRV2_DR_PDS constructs and collected 2 weeks later. Then, *pds*, *Rosea1*, and *Delila* mRNA levels were measured by quantitative reverse transcription (RT)-PCR. In parallel, anthocyanin content was determined as cyanidin-3-glucoside equivalents. Figure 1E confirms that the mRNA levels of marker genes *Delila* and *Rosea1* as well as *pds* negatively correlated with anthocyanin content in the fruit. This result confirmed that purple color (i.e. anthocyanin accumulation) in the fruit could be used as a qualitative indication of the level of gene silencing that is taking place in this fruit/tissue.

Anthocyanin-Monitored Fruit VIGS Transferred to Globe-Type Fruit

Anthocyanin-monitored VIGS confirmed that silenced and nonsilenced tissues occur within the same sample (fruit) as reported previously (Orzaez et al., 2006). This may reduce the sensitivity of VIGS in terms of identifying the effects on gene silencing, unless silenced sectors can be readily identified and analyzed independently. Dissection of silenced (red) from nonsilenced (purple) tissues is straightforward, and we thought such guided dissection should facilitate the identification of the molecular/phenotypic changes introduced by the depletion of a GOI. To facilitate further the tissue dissection and to increase yields of silenced tissue for downstream analysis, the

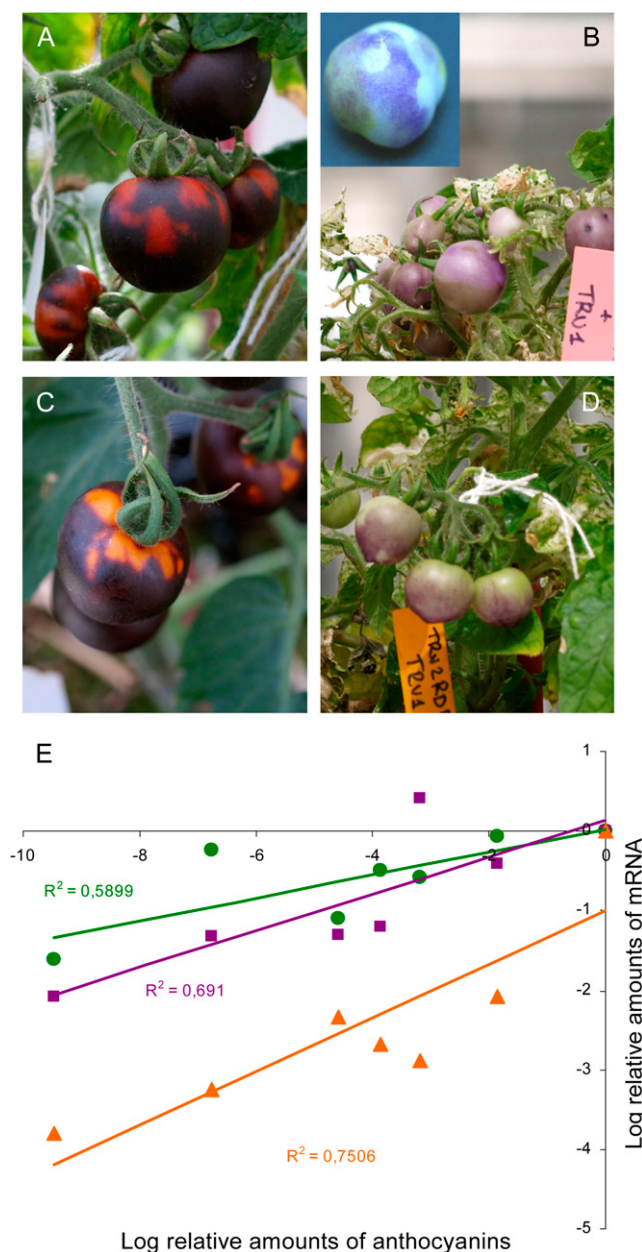


Figure 1. Silencing of *Del/Ros1* and *pds* in MT *Del/Ros1* fruit. A, Four-week-old MT plants were agroinfected with a mix of pTRV1, pTRV2_DR, and pTRV2_PDS *Agrobacterium* cultures to render systemically silenced fruit. At the ripe stage, only red or purple sectors were observed. B, Mature-green fruit from plants treated as in A showed light purple sectors, indicating *pds* but not *Del/Ros1* silencing. A close-up of a detached tomato with light purple sectors can be observed in the inset. C, Systemic VIGS agroinfiltration using pTRV1 in combination with a pTRV2_DR_PDS tandem construct produced fruit showing only purple and yellow sectors, the latter resulting from simultaneous silencing of *Del/Ros1* and *pds*. D, Mature green fruit from plants treated as in C showed either white or dark purple (resulting from purple on green background) sectors. No light purple (purple on white) sectors were detected in these plants. E, Correlations between anthocyanin content and *Rosea1*, *Delila*, and *pds* mRNA levels. Anthocyanin levels are represented as the common logarithm of the relative amounts of cyanidin-3-glucoside equivalents, setting the anthocyanin content of

Del/Ros1 module was transferred from the cherry-type MT tomato to a larger globe-type tomato background by crossing *Del/Ros1* MT with a wild-type Money-Maker (MM) plant. Plants yielding purple globe tomatoes showing a good fruit VIGS response were selected after an initial cross and self-pollination through to the F6 generation. The resulting plant line, referred to hereafter as F6DR, showed smooth leaves, indeterminate growth, and plant size intermediate between MM and MT as its main morphological characters (Fig. 2A).

The efficiency of fruit VIGS was improved substantially by the use of plants producing globe-type fruit. pTRV2_DR agroinjection yielded fruit whose silenced surface, albeit still variable, could reach up to 80% of the total fruit. Internally, silencing affected all tissues in the fruit, including pericarp, central lamella, and locular tissues (Fig. 2B). The efficiency of cosilencing using tandem constructs was also assayed in globe-fruited plants. As previously, *pds* was used as a test GOI to identify visually the extent of cosilencing in the fruit. When the pTRV2_DR_PDS construct was agroinjected into the fruit, silencing of *pds* and *Del/Ros1* was strictly colocalized, rendering fruit decorated with purple and yellow sectors only (Fig. 2C). We were also able to confirm that the fruit VIGS technique confined the silencing effect to the specific agroinjected fruit, as seen in Figure 2C. Confinement of the VIGS signal within agroinjected fruit should facilitate the design of high-throughput experiments using one fruit-one replicate rather than one plant-one replicate strategies.

Del/Ros1-Silenced Sectors of F6DR Fruit Are Largely Devoid of Transgene Influence

As opposed to endogenous VIGS reporters such as PDS, the silencing of the transgenic reporter *Del/Ros1* notionally should yield a wild-type situation where GOI function can be analyzed with minimal interference from silencing of the marker genes. To assess the extent to which the phenotypic effects of *Del/Ros1* were reverted by VIGS treatment, *Delila* and *Rosea1* mRNA levels were quantified separately in red, silenced areas (named DR_S) and purple, nonsilenced areas (named DR_NS) from pTRV2_DR-treated F6DR tomatoes. As shown in Figure 3A, *Delila* and *Rosea1* mRNA levels were significantly reduced in DR_S tissues. To characterize further the composition of DR_S tissues, phenylpropanoid profiles of methanol-extracted fruit pericarp were obtained by HPLC-MS analysis. In Figure 3, B and C, methanol extracts of DR_S samples are shown in comparison with nonsilenced pericarp from a F6DR hybrid. Phenylpropanoid profiles of

TRV1-agroinjected control *Del/Ros1* fruit as 1. The extent of gene silencing is represented as the base-two logarithm of the relative levels of *Rosea1* (circles), *Delila* (squares), and *pds* (triangles) mRNAs in MT fruit agroinjected with a combination of pTRV1 and pTRV2_DR_PDS constructs, as estimated by quantitative RT-PCR. The abundance of each mRNA in TRV1-alone agroinjected control fruit was set as 1.

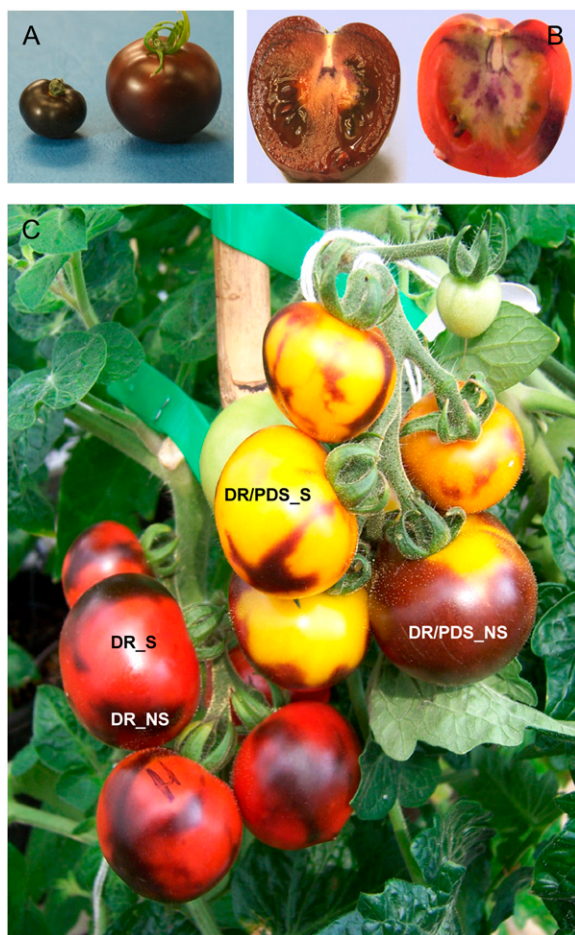


Figure 2. Anthocyanin-mediated visualization of VIGS in a globe-type fruit. A, A MT *Del/Ros1* fruit (left) compared with a purple tomato resulting from crossing MT *Del/Ros1* and wild-type MM (right). B, Cross section of a control tomato (left) and *Del/Ros1*-silenced tomato (right), the latter showing nonsilenced purple sectors and veins. C, Results from fruit VIGS on globe purple tomatoes from the same plant using pTRV2_DR/PDS (top right) or pTRV2_DR (bottom left) construct. DR_S, Silenced sectors of pTRV2_DR-treated tomatoes; DR_NS, non-silenced sectors of pTRV2_DR-treated tomatoes; DR/PDS_S, silenced sectors of pTRV2_DR/PDS-treated tomatoes; DR/PDS_NS, nonsilenced sectors of pTRV2_DR/PDS-treated tomatoes.

parental lines MT and MM are also shown for comparison. Silenced sectors were almost completely devoid of the anthocyanins abundant in *Del/Ros1* fruit (listed in Fig. 3D), showing HPLC-MS profiles that matched closely those of nontransgenic parental lines. These results indicated that, as far as phenylpropanoid content was concerned, DR_S samples were largely devoid of the influence of the transgenes.

F6DR Fruit Have Slightly Reduced Levels of Lycopene-Derived Volatiles, Which Are Reverted by *Del/Ros1* Silencing

Particularly challenging is the use of VIGS for analysis of genes affecting the fruit aroma, due to the labile

nature of volatile compounds, their dependence on the genetic background, and physiological and environmental conditions. We were also concerned that the presence of *Del/Ros1* might itself influence the volatile profile. Indeed, the GC-MS profiles of DR_S sectors from F6DR fruit were found to be statistically different from those obtained from DR_NS sectors, as evidenced by class separation in unsupervised principal component analysis (PCA; Supplemental Fig. S2A). Geranial and 6-methyl-5-hepten-2-one were found as major contributors to the observed differences (Supplemental Fig. S2C). This could imply that *Del/Ros1* expression induces a small reduction (1.6- to 1.7-fold on average; Supplemental Table S1) in the levels of lycopene-derived volatiles in F6DR fruit, which are then restored upon VIGS in DR_S sectors. An indication in this direction, despite representing a different genetic background, comes from the analysis of *Del/Ros1* effect in the volatile profiles of MT plants. When transgenic MT *Del/Ros1* plants were compared with their wild-type MT isogenic line, differences were observed between classes (Supplemental Fig. S2B), with volatile changes, particularly geranial and 6-methyl-5-hepten-2-one, following similar trends as in F6DR fruit (Supplemental Fig. S2D). We concluded that in F6DR, the presence of the *Del/Ros1* transgenic module had a measurable influence on the volatile profile of the fruit, with this influence being to a large extent eliminated in DR_S sectors. This effect precluded us from using direct within-fruit comparisons (DR/GOI_S versus DR/GOI_NS) in AMFV functional analysis of volatile-related genes. Instead, a general between-fruit analytical strategy (DR_S versus DR/GOI_S) was adopted to extract maximum information about the effects of GOI silencing on volatile profiles.

VIGS Analysis of Tomato Volatile Profiles: Effects of *pds* Silencing

Ultimately, the capacity of AMFV for extracting functional information on genes involved in metabolic (volatile) profiles needs to be tested experimentally with candidate genes whose silenced phenotype subsequently can be validated by other means. To test the reliability of the *Del/Ros1* marker technology, we chose to analyze the effects of silencing the *TomloxC* and *pds* tomato genes. *TomloxC* RNA interference has been shown to reduce the production of volatile aldehydes in tomato fruit (Chen et al., 2004a). On the other hand, *pds* VIGS renders an easily scorable phenotype, allowing comparisons of VIGS in wild-type and *Del/Ros1* backgrounds.

As a first proof of concept, the effects of *pds* silencing on the fruit volatile profile were analyzed. Fruit were agroinjected with the pTRV1 vector combined with pTRV2_DR or pTRV2_DR_PDS plasmids. Three weeks after infiltration, fruit showing good VIGS responses (50% silenced surface on average) were carefully selected, harvested, and processed. Volatile profiles from silenced tissues were compared using unsupervised

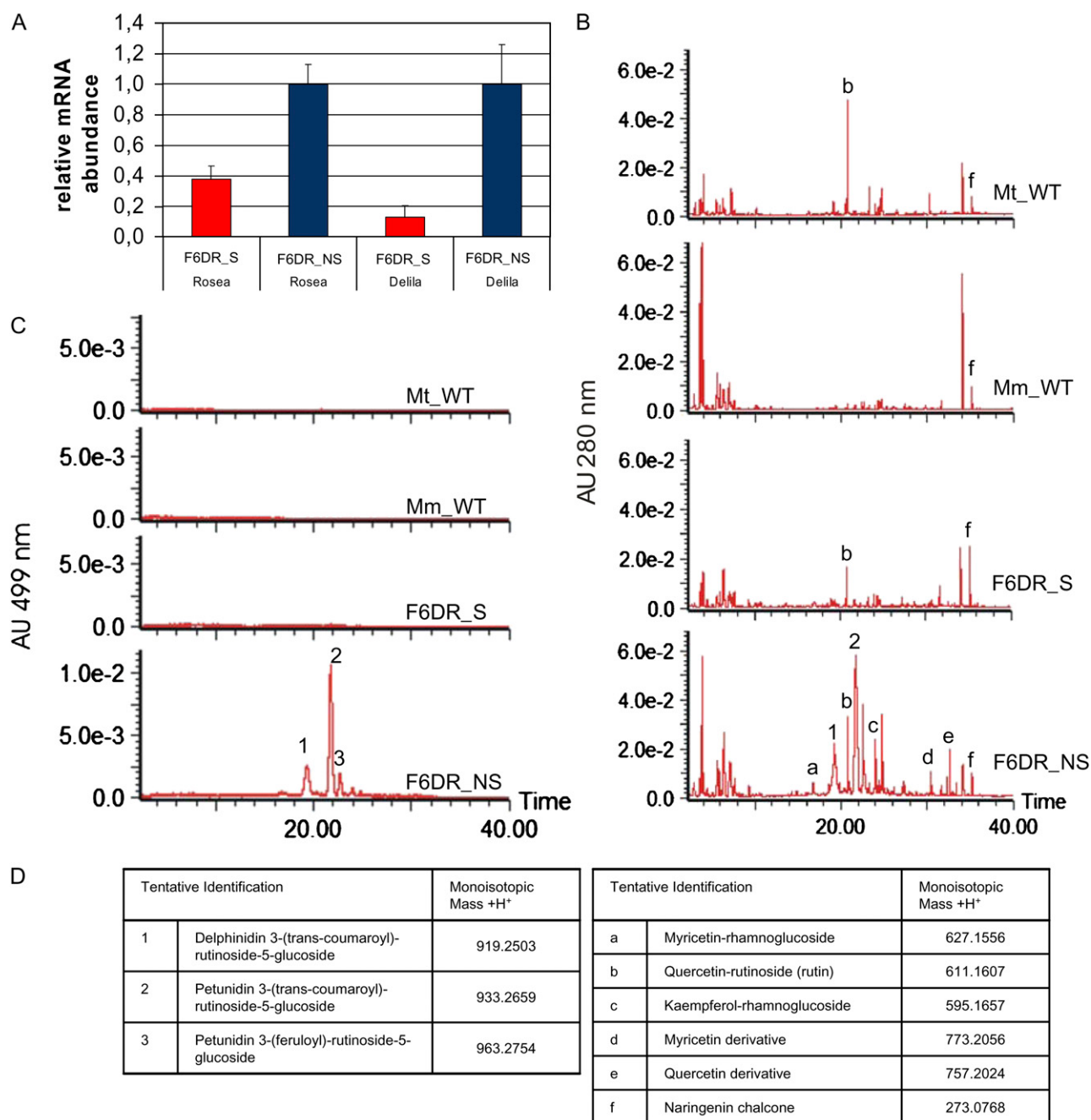
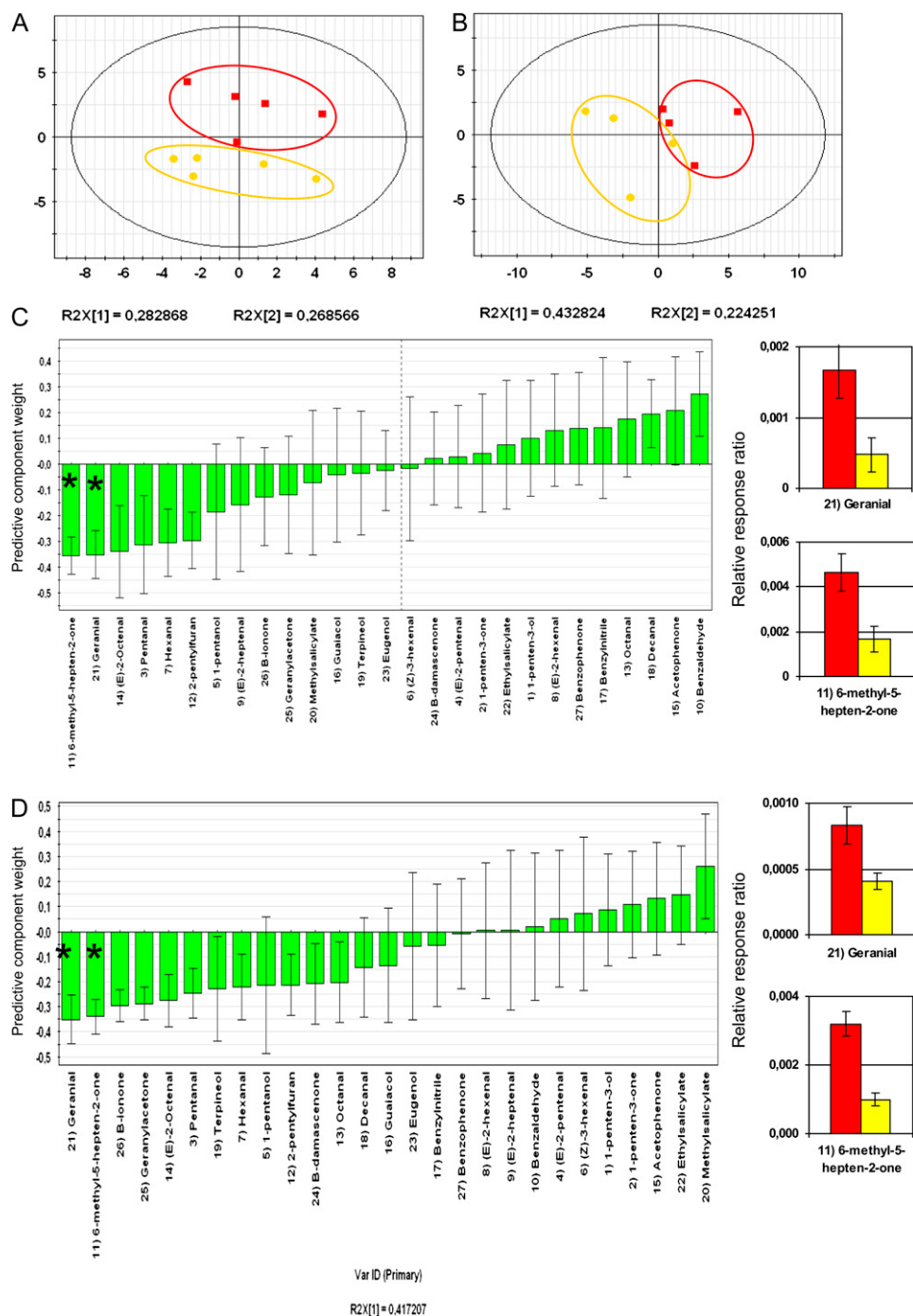


Figure 3. VIGS-mediated reversion of anthocyanin accumulation in red sectors of globe-type tomatoes. A, Relative mRNA levels of *Rosea1* and *Delila* in red and purple sectors of F6DR tomatoes, with levels in purple sectors set as 1. B and C, UPLC analysis as profiled at 280 and 499 nm, respectively, of methanol extracts from untransformed MT pericarp (Mt_WT), untransformed MM pericarp (Mm_WT), DR_S pericarp sectors from line F6DR (F6DR_S), and DR_NS sectors from line F6DR (F6DR_NS). D, The putative identity of the main UPLC picks is shown in profiles B and C. [See online article for color version of this figure.]

PCA. As shown in Figure 4A, PCA separated samples from the *pds* experiment into two groups, which coincided with the experimental classes DR/PDS_S and DR_S. Separation between classes occurred mainly along the *y* axis, explaining 26% of the total variance. As a strategy for identification of the metabolites contributing to the differences between classes,

orthogonal partial least-squares latent structures discriminate analysis (OPLS-DA) was performed. OPLS-DA was visualized in the weighting plot shown in Figure 4C, representing the contribution (X weight) of each metabolite to class separation, with positive and negative values representing up-regulation and down-regulation behavior, respectively. Jack-knifed confi-

Figure 4. Volatile analysis of *pds*-silenced tomatoes. A, Unsupervised PCA of volatile compounds detected in DR_S (red dots) and DR/PDS_S (yellow dots) tomato samples using AMFV. B, PCA of volatile compounds detected in yellow and red sectors from MM tomatoes silenced in *pds* using unmonitored fruit VIGS. C, Loading plot of OPLS-DA with jack-knifed bars for the same data set as in A. D, OPLS-DA loading plot from the MM *pds* experiment. Columns represent the weight of the contribution of each metabolite to the discrimination between classes. Jack-knifed bars represent 95% confidence intervals. Detailed quantification from 6-methyl-5-hepten-2-one and geranial (labeled with asterisks) is represented in yellow (*pds*-silenced sectors) or red (*pds*-nonsilenced sectors) in each experiment. Biologically independent samples were analyzed in triplicate. Columns represent relative abundance of each metabolite to total ion content $\pm$ SE.



dence intervals reflect the reliability for each loading value (Kruger et al., 2008; Wiklund et al., 2008). Only the two main products of lycopene degradation (geranial and 6-methyl-5-hepten-2-one) combined high covariance and narrow confidence intervals (Fig. 5C). Detailed quantification of each metabolite is also shown; a dramatic and highly significant reduction in geranial and 6-methyl-5-hepten-2-one levels was observed upon *pds* silencing ($P < 0.0005$ for both compounds; Supplemental Table S1).

The effects of *pds* silencing on the aroma profile were also evaluated in a wild-type background (MM). In this case, the visually scorable PDS phenotype allowed the dissection of the fruit into silenced and nonsilenced sectors. Therefore, comparisons between data obtained with *pds* in wild-type and *Del/Ros1* backgrounds can be indicative of the robustness of the *Del/Ros1* tracking system. Mature green MM tomatoes where agroinjected with pTRV2_PDS constructs. Three weeks after treatment, yellow, *pds*-silenced sec-

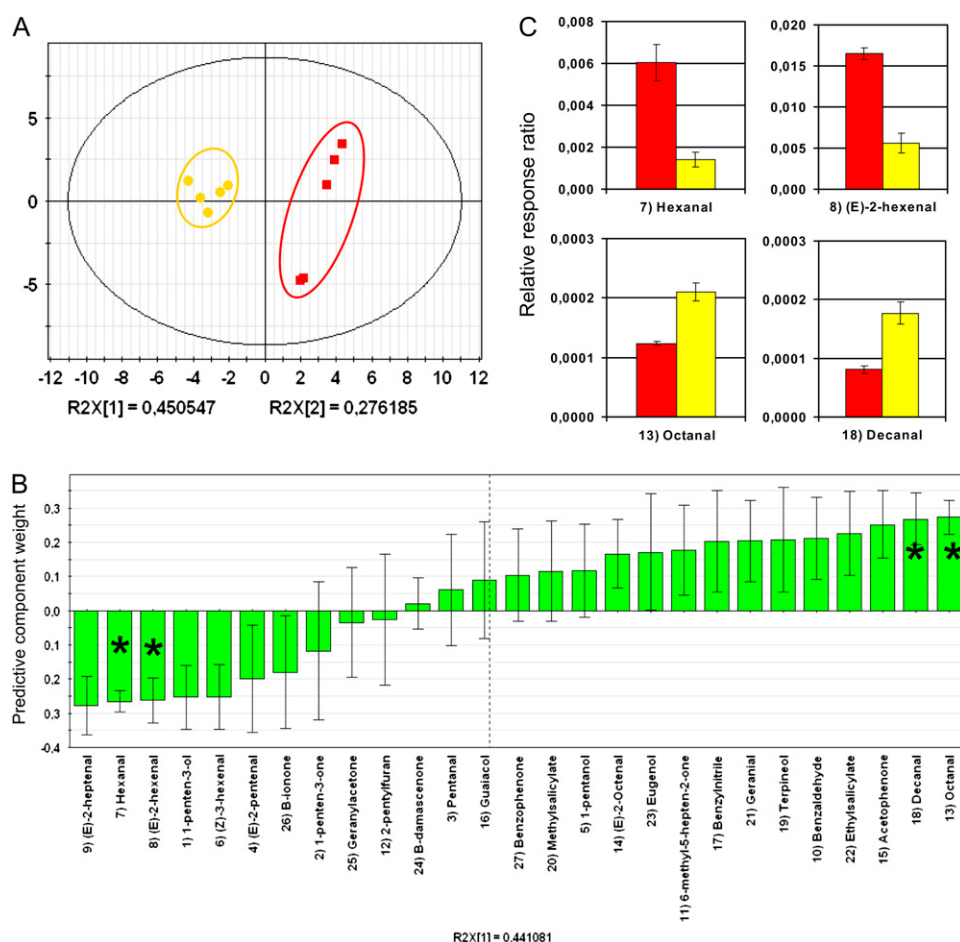


Figure 5. Volatile analysis of *TomloxC*-silenced tomatoes. A, Unsupervised PCA generated from GC-MS data from DR_S (red dots) and DR/LXC_S (yellow dots) tomato samples. B, Loading plot of the OPLS-DA model for the *TomloxC* experiment. Columns represent the weight of the contribution of each metabolite to the separation in the predictive component. Jack-knifed bars represent 95% confidence intervals. C, Detailed quantification of metabolites combining highest contribution and narrowest confidence intervals (labeled with asterisks). Columns represent relative abundance of each metabolite to total ion content $\pm$ SE. A minimum of five biologically independent samples were analyzed in triplicate for each category.

tors were dissected from red, nonsilenced sectors and processed. As shown in Figure 4B, PCA also separated samples between *pds* silenced and nonsilenced. As in the AMFV *pds* experiment, geranial and 6-methyl-5-hepten-2-one were the only two volatiles showing highly significant changes ($P < 0.0001$), also appearing in the OPLS-DA loading plot (Fig. 4D), confirming that the AMFV-observed changes were genuine effects of *pds* silencing. Interestingly, less significant effects were also observed for other carotenoid-derived compounds in MM *pds*-silenced tomatoes, which had no counterpart in AMFV experiment (Supplemental Table S1). These differences probably can be attributed to the varietal differences in aroma composition between F6DR and MM or to differences in sampling with respect to developmental timing and/or environmental differences between experiments.

VIGS Analysis of Tomato Volatile Profiles: Effects of *TomloxC* Silencing

As a second proof of concept, the effects of *TomloxC* silencing on the fruit volatile profile were analyzed in a separate experiment. A 242-bp cDNA fragment showing low identity (<50%) to other tomato lipoxygenases was introduced into pTRV2_DR_GW, yielding

the pTRV2_DR_LXC vector. Subsequently, fruit were agroinjected with the pTRV1 vector combined with pTRV2_DR or pTRV2_DR_LXC plasmid. Volatile profiles from silenced tissues were compared using unsupervised PCA. As shown in Figure 5A, PCA separated samples from the *TomloxC* experiment into two groups, which coincided with the experimental classes DR/LXC_S and DR_S. Separation between classes occurred mainly along the x axis, explaining 46% of the total variance. It was found that the major contributors to DR/LXC_S versus DR_S separation were C-6 aldehydes derived from fatty acid degradation, mainly hexanal but also *E*-(2)-hexenal and *Z*-(3)-hexenal, which combined highest covariance and narrowest confidence intervals. Also, C-5 aldehydes were identified as relevant down-regulated metabolites. On the up-regulated side of the plot, decanal and octanal, also potentially derived from fatty acid degradation, were also identified in the analysis (Fig. 5B). Integration data for the most relevant metabolites are also shown in Figure 5C. A severe and highly significant reduction in C6 and C5 aldehyde levels was observed upon *TomloxC* silencing ($P < 0.0005$ for hexanal), whereas octanal ($P < 0.0002$) and decanal ($P < 0.0007$) increased in abundance significantly (Supplemental Table S1).

Silencing of Tomato ODO1 Modifies the Profile of Benzenoid Volatile Compounds in Fruit

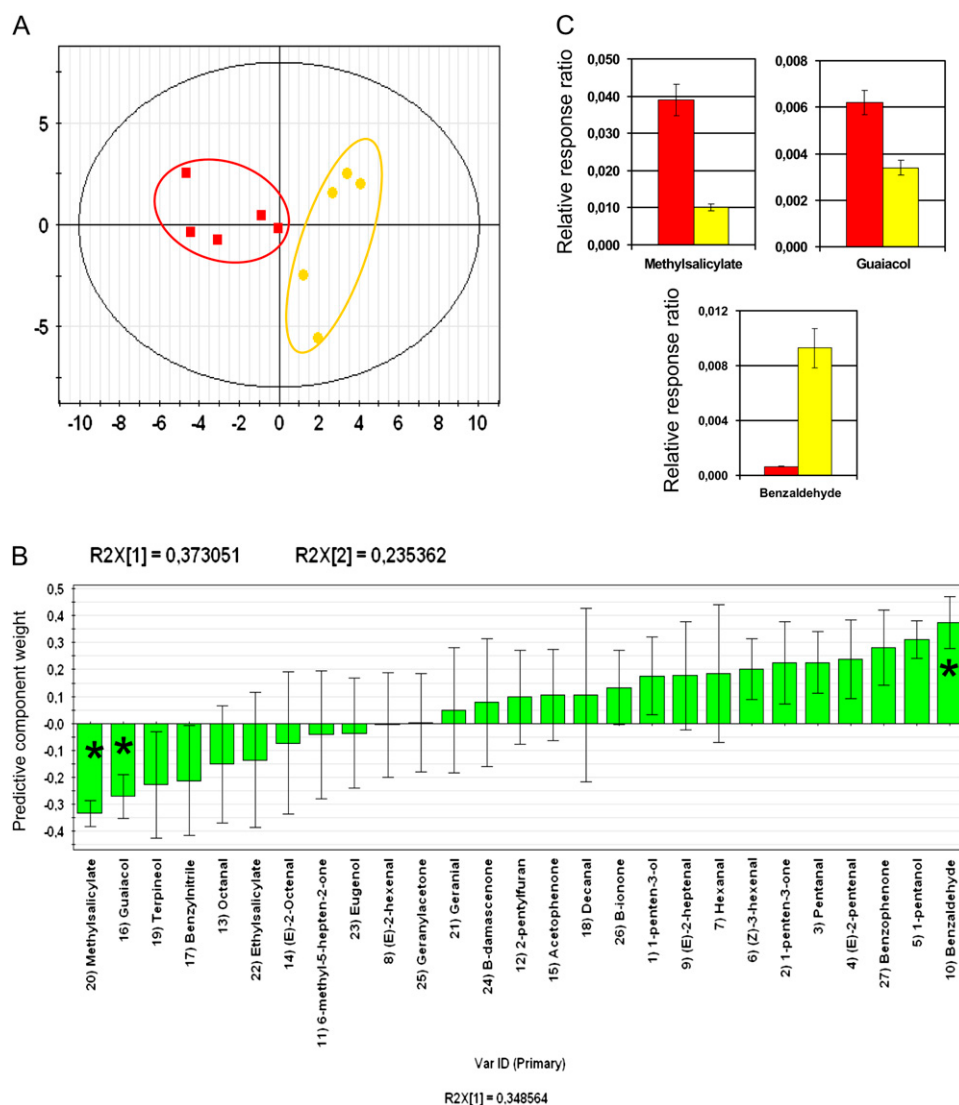
BLAST analysis of tomato expression databases using petunia *ODO1* cDNA as bait identified a tomato unigene, SGN-U344597, which showed 67% identity with its petunia homolog. A single EST, SGN-U344597, was isolated from a fruit library. Based on this sequence, two oligonucleotides were designed to isolate a fragment of 185 bp of the putative tomato *ODO1* homolog (*SlODO1*), corresponding to nucleotides 474 to 688 of the 783-bp open reading frame. BLAST analysis performed using a 185-bp fragment as bait did not identify additional *SlODO1*-like genes other than SGN-U344597 in the fruit. The effect of *SlODO1* depletion on fruit aroma was tested using AMFV, following the same procedure as described above. *SlODO1* silencing had a clear influence on the volatile profile, as evidenced by the grouping of samples shown in the PCA scores plot in Figure 6A. OPLS-

DA of the same samples showed significant differences in benzenoid-derived compounds, with a decline of methyl salicylate and guaiacol levels in silenced tissues and, most notably, a steep increase of benzaldehyde, whose levels increased, on average, 8-fold in *SlODO1*-silenced tissues (Fig. 6, B and C). Also, the group of C5 volatiles [pentanal, (*E*)-2-pentenal, 1-penten-3-one, and 1-pentanol] showed consistent increases in silenced areas.

DISCUSSION

Fruit VIGS in tomato has been shown to be useful in addressing gene function involving visually scorable phenotypes (Xie et al., 2006). However, its irregular distribution and efficiency, which are known to be strongly influenced by environmental factors such as temperature and humidity (Fu et al., 2006), complicate its use for finding links between metabolic and genetic

Figure 6. Volatile analysis of *SlODO1*-silenced tomatoes. A, Un-supervised PCA generated from fruit GC-MS data from DR_S (red dots) and DR/ODO_S (yellow dots) tomato samples. B, Weightings plot of the OPLS-DA model for the *SlODO1* experiment. Jack-knifed bars represent 95% confidence intervals. Columns represent the weight of the contribution of each metabolite to the discrimination between classes. C, Detailed quantifications of benzaldehyde, guaiacol, and methyl salicylate in DR_S (red) and DR/ODO_S (yellow) sectors. Columns represent relative abundance of each metabolite to total ion content $\pm$ SE. A minimum of five biologically independent samples were analyzed in triplicate for each category.



data without an associated visual marker (D. Orzaez and A. Granell, unpublished data). The phenotypic reversion obtained by silencing *Delila* and *Rosea1* genes in *Del/Ros1* fruit suggested that the depletion of anthocyanin content could serve as an internal marker for VIGS. However, to function as a reporter system, the simultaneous silencing of the marker genes and GOIs needed to be ensured; therefore, the requirements for simultaneous silencing of at least two genes in tomato needed to be established. In *N. benthamiana*, the silencing signal expands beyond the infective wave of the virus through a silencing amplification mechanism. This mechanism works independently of the presence of the virus, allowing the silencing wave to move ahead of the virus infection wave (Ruiz et al., 1998). In *N. benthamiana*, silencing extends almost uniformly; consequently, the extent of VIGS symptoms does not correlate with the abundance of the virus (Rotenberg et al., 2006). Since there are no reports showing mutual exclusion of different silencing signals within the same plant tissues, we thought it possible that silencing signals initiated concomitantly at the inoculation site in tomato by simultaneous agrodelivery of pTRV2_DR and pTRV2_GOI could result in cosilencing of all three genes in distal parts of the plant. However, this was not the case for tomato. A possible explanation is that the spread of the silencing signal in tomato needs to be assisted by the virus. Contrary to the situation in *N. benthamiana*, the severity of TRV-induced silencing symptoms in tomato leaves positively correlates with the amount of virus RNA, indicating that the silencing signal runs parallel with the infective wave and that close proximity of cells to the virus is required for the silencing mechanism to work efficiently (Rotenberg et al., 2006). Since viral movement through the plant is often a stochastic process subjected to complex mechanisms and affected by environmental factors, sink-source relationships, tissue age, clone competition, and others, the patchy penetration of the VIGS phenotype found in tomato and other plant species could be the result of irregular viral distribution throughout the plant. Clonal exclusion between coinoculated viruses could also explain why codelivery of VIGS constructs is not sufficient for inducing simultaneous silencing of two or more genes in tomato. Establishing the relationship between viral loading and VIGS response will help to clarify the mechanisms and to optimize VIGS efficiency in different crop plants.

In contrast to codelivery, we showed that the tandem arrangement of up to three different target sequences within the same VIGS vector provides a highly efficient way of achieving simultaneous silencing. The use of *pds* as a model GOI produced a visual silencing pattern in fruit that demonstrated this point clearly. Furthermore, transferring the *Del/Ros1* phenotype to larger globe-type fruit facilitated tissue dissection and yields required for downstream analysis by metabolite profiling. Using these refinements to the AMFV method, we have tested the extent to which

functional information can be extracted by comparing the volatile profiles of silenced and nonsilenced samples for two candidate genes involved in aroma formation in tomato fruit.

AMFV was tested using two genes, *pds* and *TomloxC*. Switching off metabolic flux by reducing *pds* or *TomloxC* levels modified the general volatile profile, as shown by class separation observed in unsupervised PCA visualization. Most convincingly, in both examples, OPLS-DA visualization tools correctly pinpointed, from among the whole set of metabolites profiled by GC-MS, those most closely related to the metabolic step affected. OPLS has been shown previously to be a convenient tool for identification of biochemically interesting compounds in GC-MS-based metabolomics data (Wiklund et al., 2008), and our results confirm this point.

Chemometric analysis of tomato volatiles from *pds*-silenced fruit sectors clearly identified highly significant differences in 6-methyl-5-hepten-2-one and geranial. These two compounds are breakdown products of lycopene and were earlier shown to be depleted in carotenoid biosynthetic mutants with reduced lycopene content (Lewinsohn et al., 2005a, 2005b). These results were corroborated with a more traditional VIGS analysis carried out in wild-type MM, taking advantage of the visual phenotype provided by *pds* silencing. Volatiles 6-methyl-5-hepten-2-one and geranial were again the most severely depleted volatile compounds in yellow sectors of MM tomatoes, confirming the results obtained by AMFV. This result is particularly remarkable and provides an example of how metabolic interference is easily overcome in AMFV analysis. As deduced from the volatile profile of *Del/Ros1* MT fruit and from direct DR_S versus DR_NS comparisons in F6DR fruit, the levels of lineal carotenoid-derived volatiles are reduced 1.6- to 1.7-fold in anthocyanin-rich tissues. Consequently, the silencing of the GOI and the silencing of the marker *Del/Ros1* module push the levels of carotenoid-derived volatiles in opposite directions. This interaction could reasonably be expected to interfere with the analysis of any candidate gene involved in the production of a particular metabolite, therefore masking its effects. However, using DR/GOI_S versus DR_S comparisons instead of DR/GOI_S versus DR/GOI_NS ones provides a means of circumventing any interference in metabolite levels resulting from *Del/Ros1* activity. This was demonstrated clearly in the case of lycopene-derived volatiles in the *pds*-silencing experiment.

Other volatiles that were reduced by *pds* AMFV, albeit less significantly, were also reduced in regular VIGS experiments (Fig. 4, compare C and D). These second-level changes are probably the result of the cross talk between metabolic pathways in the complex metabolic networks of ripening fruit and may provide additional clues to how such pathways interact. Conversely, β -carotene-derived volatiles, β -ionone and β -damascenone, exceptionally showed different behavior in the two VIGS experiments, with no changes

in AMFV and significantly lower levels in *pds*-silenced MM samples. We think that the observed differences may be due to the different genetic backgrounds of the tomato lines and their respective developmental rates. There is evidence that β -carotene pools are synthesized in the fruit before ripening, at least in certain genetic backgrounds (Tieman et al., 2006). Fruit VIGS experiments, which are initiated at the mature-green stage, may or may not block pre-ripening synthesis of β -carotene depending on the speed at which silencing signals spread through the fruit with respect to the precise developmental stage in which biosynthesis takes place, which may be heavily influenced by the genetic background. The fact that a strong genetic link is often observed between β -ionone and β -damascenone in quantitative trait locus analysis, but not between these two compounds and lycopene-derived volatiles, strongly reinforces this interpretation (Mathieu et al., 2009).

Antisense and cosuppression-mediated depletion of *TomloxC* in stable transgenic plants has been reported previously (Chen et al., 2004a). *TomloxC*-silenced fruit were shown to contain lower levels of C-6 volatile compounds, consistent with *TomloxC* encoding a 13-lipoxygenase that catalyzes hydroxyperoxidation of linoleic and linolenic acids to yield the precursors of C-6 volatiles. The same conclusion was drawn from anthocyanin-guided VIGS analysis in a 5-week-long experiment using no more than 30 fruit. *TomloxC*-silenced fruit sectors were depleted in C-6 but also in C-5 volatiles, the latter probably derived from C-6 breakdown. Interestingly, OPLS-DA also identified decanal and octanal metabolites as showing significant increases in levels. An increased conversion of the *TomloxC* substrate, linoleic acid (C-18), into C-8 and C-10 compounds in silenced tissues could explain these increases. Whether this conversion takes place in tomato as a result of a specific enzymatic activity remains to be established.

Finally, AMFV was used for functional analysis of the tomato homolog of the petunia R2-R3 myb factor ODO1 in fruit. In petunia, *ODO1* controls the synthesis of benzenoid volatiles via the up-regulation of the expression of several enzymes in the shikimate pathway leading to benzenoid volatiles, with the significant exception of benzoic acid methyl transferase (Verdonk et al., 2005). The effect of *ODO1* in petunia was confirmed using a color-assisted VIGS methodology, using the endogenous chalcone synthase gene as a marker for VIGS (Spitzer et al., 2007). We think that the transgenic strategy proposed here may reduce the interference and therefore widen the range of applications of marker-assisted VIGS, since it operates through a phenotype that reverts to the wild type rather than through a mutant, metabolite-depleted phenotype. For example, VIGS could be monitored in leaves or shoots in plants transformed with *Delila* and *Rosea1* driven by leaf- or shoot-specific promoters without the impact of loss of function of the marker genes.

The target gene regulation of specific myb genes cannot always be directly translated from one plant species to another based on sequence homology, as has been repeatedly observed (Martin and Paz-Ares, 1997; Mehrtens et al., 2005; Luo et al., 2008). *ODO1* VIGS in petunia resulted in lower levels of almost all analyzed benzenoid compounds. As expected, in tomato fruit, benzenoid volatiles were also severely affected by the silencing of *SlODO1*, confirming the role of this regulatory gene in modulating benzenoid metabolism in tomato. However, according to our AMFV data, *SlODO1* does not seem to activate the whole pathway in tomato but rather modulates the relative abundance of different intermediates in methyl benzoate synthesis, presumably through regulating expression of only some target genes operational in the pathway. This is, to some extent, also true in petunia, where benzoic acid methyl transferase does not appear to be activated by *ODO1* and, in fact, shows increased expression in response to *ODO1* silencing. In tomato, the accumulation of high levels of benzaldehyde in combination with reduced levels of methyl salicylate and guaiacol in response to *SlODO1* silencing may indicate that *SlODO1* regulates flux between benzoic acid and its methylated/hydroxylated derivatives. Additional experiments are required to establish the precise targets of *SlODO1* in tomato fruit. The functional data provided by AMFV provide a first line of evidence of its regulatory role in tomato and material for analysis of target gene expression.

In conclusion, we present a VIGS tool for tomato that enables studies of gene function for genes active in fruit ripening that, by themselves, would not produce a visual phenotype. We have tested successfully three tomato genes encoding two enzymes and one transcription factor, influencing the three main routes of volatile production in tomato (i.e. carotenoids, fatty acids, and phenylpropanoids) using AMFV analysis. This technology provides a means for rapid, high-throughput analysis of genes involved in all of the metabolic networks underlying tomato fruit ripening and associated nutritional and health-related quality traits.

MATERIALS AND METHODS

Plant Materials

Tomato (*Solanum lycopersicum*) MT plants transformed with *Delila* and *Rosea1* cDNAs under the control of the E8 ripening-specific promoter were used as an initial source of anthocyanin-accumulating fruit (Butelli et al., 2008). Globe-type purple tomatoes were obtained by crossing *Del/Ros1* MT N line (T2 homozygous generation) with wild-type MM plants. Segregating sibling lines were self-pollinated and selected through to the F6 generation on the basis of their phenotypic characteristics (i.e. globe-type fruit, smooth leaves, indeterminate growth, and best fruit VIGS response). The quality of VIGS response in each line was evaluated from the percentage of fruit agroinjected with the pTRV2_DR construct that developed red sectors and the size of the red sectors with respect to the total fruit surface. Plants were grown in a greenhouse supplemented with artificial light as described earlier (Marti et al., 2007).

Cloning Procedures and Vector Construction

TRV-based silencing vectors pTRV1 and pTRV2 were kindly provided by Prof. Dinesh Kumar (Liu et al., 2002). A pTRV2_DR construct was generated by introducing a 284-bp *EcoRI/XhoI* DNA fragment (DR fragment) into pTRV2 vector. The DR fragment was obtained from the PCR fusion of D and R fragments. The D fragment comprises 129 bp of the *Delila* gene and was PCR amplified from *Del/Ros1* tomato genomic DNA using oligonucleotides D06SEP02DEL1F (5'-TGGCGGCGGCGGCGGTCCAATTCAGTTGCACAAC-CAGG-3') and D06SEP04DEL1R (5'-CACCGAATTCACCTTCTCTGCAATCCACAGTGATC-3'). The R fragment was obtained from the same source using oligonucleotides D06SEP03ROS1R (5'-CCGCCGCCGCCCAAGTACGTGCAATCCGGTGA-3') and D06SEP01ROS1F (5'-GGGGCTCGAGTC-TAGATGTGGGAAGAATTTAGGCGAGG-3'). The D and R fragments were gel purified and fused in an overlapping PCR (15 cycles) with oligonucleotides D06SEP01ROS1F and D06SEP04DEL1R. A pTRV2_DR_GW destination vector was generated by linearizing pTRV2_DR with *SmaI* followed by blunt-end cloning of a Gateway Destination Cassette (*rfa_verB*) with a Gateway Vector Conversion Kit (Invitrogen) following the manufacturer's instructions. For the pTRV2_DR_PDS construct, a 409-bp PDS fragment between oligonucleotides S07FEB01PDSF1 (5'-GGCACTCAACTTTATAAACC-3') and S07FEB02PDSR1 (5'-GGTTTGACAGAAAAGTGAAG-3') was first cloned into the pCR8/GW-TOPO vector and transferred by LR recombination into the pTRV2_DR_GW destination vector. Similarly, pTRV2_DR_ODO and pTRV2_DR_LXC were constructed from DNA fragments cloned in pCR8/GW-TOPO using oligonucleotides A07NOV01 (5'-GGTTGTACCGGAGAG-TACAAGCG-3') and A07NOV05 (5'-CCACTCCTTAACAGCCTACTGGA-ATC-3') for ODO1 and A07NOV03 (5'-CTCCGGCAACACCGTTTACTCC-GCCC-3') and A07NOV11 (5'-GCTCTTTGAATCCCTCAATTTGGTCC-3') for LOXC, respectively.

Plant Treatments

Unless otherwise stated, all plant inoculations were performed using 1:1 (v/v) mixtures of two C58 strain *Agrobacterium tumefaciens* cultures, one containing the pTRV1 vector and the second containing a pTRV2-derived vector. Bacterial clones were grown overnight in liquid medium, then transferred to infiltration medium as described earlier (Orzaez et al., 2006). For coinfiltration studies, 1:1:1 mixes of pTRV1, pTRV2_GOI, and pTRV2_DR were used. Inoculations intended for systemic VIGS were performed by syringe agroinfiltrations of *Agrobacterium* suspensions (optical density at 600 nm = 0.5) in cotyledons of 3- to 4-week-old plants. In fruit-VIGS, flowers were labeled at anthesis and inoculated 4 weeks later (mature green stage) by agroinjecting 0.5 mL of bacterial suspension (optical density at 600 nm = 0.05) through the peduncle into the fruit. Agroinjected fruit were tagged at breaker (turning) stage and collected, unless otherwise stated, between 15 and 20 d later for further analysis.

Estimations of Anthocyanin Content and mRNA Quantification

Anthocyanin content in fruit extracts was estimated as cyanidin-3-glucoside equivalents as described earlier (Lee et al., 2005). The relative abundance of *pds*, *Rosea1*, and *Delila* mRNAs in pericarp samples was determined by quantitative RT-PCR. RNA samples from tomato pericarp were prepared with an RNeasy Plant Mini Kit using on-column RNase-free DNase Set treatment (Qiagen) and copied to cDNA with SuperScript II reverse transcriptase (Invitrogen). Primers used were PDSF1 (5'-TCATCAACCTTCCGTGCTTC-3') and PDSR1 (5'-AACATCCCTTGCCCTCAGC-3') for *pds* quantification, ROS1F (5'-ATCAAGTTCACACAGAGCAGG-3') and ROS1R (5'-CAGATAATCAACCACCTCAGCC-3') for *Rosea1* quantification, and DEL1F (5'-GGAGACATAGTTGGGAATCTTGG-3') and DEL1R (5'-AGTATGGCTA-TGCAACGCTCATC-3') for *Delila* quantification. Primers were mixed with SYBR GREEN PCR Master Mix (Applied Biosystems) in the appropriate proportions. A tomato ubiquitin amplicon was used as an internal standard for quantifications. Samples were amplified in triplicate with an ABI PRISM 7000 Sequence Detection System and analyzed with ABI PRISM 7000 SDS software.

UPLC-MS Analysis

Frozen powder (500 mg) of tomato pericarp (including the peel) was extracted with 1.5 mL of cold (−20°C) methanol acidified with 0.125% formic

acid in an ultrasound bath for 30 min. After 10 min of centrifugation at 16,000g, the supernatant was filtered through a 0.2-μm Anotop 10 membrane filter (Whatman) and analyzed by UPLC-MS using an ACQUITYUPLC-PDA system coupled to a Q-TOF Micromass spectrometer (Waters). Separation was performed on an ACQUITY BEH C18 column (150 × 2.1 mm i.d., 1.7 μm). The mobile phase consisted of 0.1% formic acid:ultrapure water (1:1,000, v/v; phase A) and formic acid:acetonitrile (1:1,000, v/v; phase B). Gradient conditions were as follows: 95% to 90% A in 14 min, 90% to 80% A in 15 min, 80% to 65% A in 10 min, 65% to 57% A in 1 min, 57% to 0% A in 1 min, held at 100% B for 3 min, returned to 95% A in 1 min, and equilibrated for 4 min before the next injection. The flow rate was 0.4 mL min^{−1}; the column and sample temperatures were kept at 40°C and 20°C, respectively; the sample injection volume was 4 μL. UV spectra were acquired between 210 and 500 nm with a 1.2-nm resolution and 20 points s^{−1} sampling rate. MS analysis was performed by electrospray ionization in positive mode. The mass spectrometer was calibrated with sodium formate (10 ng μL^{−1} in 90:10 propan-2-ol: water). Analysis conditions were as follows: capillary voltage, 3.0 kV; cone voltage, 30 eV; desolvation temperature, 300°C; source temperature, 120°C; cone gas flow, 50 L h^{−1}; desolvation gas flow, 650 L h^{−1}; collision energy, 10 eV. MS data were acquired in centroid mode in the mass-to-charge ratio scan range 100 to 1,400 with a scan time of 0.3 s and an interscan time of 0.1 s. Leu enkephalin was used as the lock mass using a LockSpray exact mass ionization source.

GC-MS Profiling and Data Analysis of Tomato Volatiles

Volatile profiling of fruit samples was carried out by Headspace SPME-GC-MS. Ground, frozen tomato pericarp (300 mg) was incubated at 37°C for 10 min in a water bath followed by addition of 300 μL of an EDTA-NaOH solution (100 mM EDTA, pH 7.5) and 0.67 g of solid CaCl₂·2H₂O to terminate endogenous enzyme activity. Samples were then sonicated for 5 min in an ultrasound bath, and a 0.7-mL aliquot was transferred to a 10-mL crimp-cap vial. Volatile analysis was performed on a 6890N Agilent gas chromatograph coupled to a 5975B inert XL MSD mass spectrometer (Agilent Technologies). Volatiles were extracted and injected automatically by means of a CombiPAL autosampler (CTC Analytics). After incubating the samples for 10 min at 50°C and continuous agitation (500 rpm), volatile compounds were extracted by exposing a 65-μm polydimethylsiloxane-divinylbenzene fiber (Supelco) to the vial head space for 10 min under the same conditions (50°C, 500 rpm). Volatiles were desorbed in the injection port of the gas chromatograph for 1 min at 250°C in the splitless mode. Separation was performed on a DB-5ms column (60 m × 0.25 mm i.d., 1-μm film thickness; J&W Scientific). Helium was used as the carrier gas at a flow rate of 1.2 mL min^{−1}. The temperature program started at 35°C for 2 min, followed by a 5°C min^{−1} ramp to 250°C, with a 5 min hold at 250°C. Mass spectra were obtained at an ionization energy of 70 eV and a scan speed of 7 scans s^{−1}, with a mass-to-charge ratio scan range of 35 to 220. Compound identification was made by comparison of GC retention time and mass spectra with those of authentic standards. Chromatograms were analyzed using the MSD ChemStation Data Analysis software (Agilent Technologies). For quantification, a representative ion peak of each compound was integrated and the area was normalized relative to the total chromatogram area.

Statistical Data Analysis

For PCA and OPLS-DA on the volatile profiles of tomato fruit, variables were centered (subtracting the average) and scaled to unit variance using the software package SIMCA-P 11 (Umetrics). The *t* tests were performed using the Microsoft Excel software package. Correlations were analyzed using Pearson's algorithm with the statistical package SPSS 15.0 (SPSS).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. VIGS-directed reversion to wild-type phenotype in *Del/Ros1* MT fruit.

Supplemental Figure S2. DR effect on volatile profile.

Supplemental Table S1. Metabolite list and significance analysis.

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